APPLIED MICROBIOLOGY, July 1974, p. 91-94

Vol. 28, No. 1
Printed in U.S.A.

Use of Sodium Polyanethol Sulfonate in the Preparation of 5% Sheep Blood Agar Plates

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Received for publication 29 April 1974

An alternative method to defibrinating sheep blood for use in bacteriological media is described. The new procedure incorporates sodium polyanethol sulfonate in a concentration of 0.05% (vol/vol). In testing 117 bacterial and fungal isolates, no significant differences were found with respect to adequate growth, pigment production, hemolytic reactions, and other physical attributes. Further tests demonstrate that the sodium polyanethol sulfonate in sheep blood agar plates does not cause any aberrations in zone sizes around disks used in antibiotic susceptibility tests. Consequently, the method represents a suitable alternative to the use of defibrinated sheep blood in the preparation of bacteriological media.

The acceptability of defibrinated sheep blood in the manufacture of blood agar plates for routine bacteriological use is well known. The earlier use of outdated anticoagulated human blood has been generally abandoned due to the inhibitory substances found in these blood preparations (1, 2). Consequently, defibrinating sheep blood has become the method of choice. However, the procurement of satisfactory defibrinated sheep blood for the manufacture of blood agar plates in the laboratory occasionally presents a problem. At times, the blood is hemolyzed or partially coagulated due to inadequate or poorly supervised defibrination procedures. Since our bacteriology laboratory uses the anticoagulant sodium polyanethol sulfonate (SPS) for routine blood cultures, we decided to investigate the possibility of utilizing SPS for the preparation of 5% sheep blood agar plates.

Our initial concern was whether blood prepared in this way would be in any way detrimental to the growth or physical characteristics of organisms isolated in a clinical laboratory. We therefore tested a large number of clinical isolates on both defibrinated blood and SPS blood agar plates to see if colonial morphology, pigment production, hemolytic reactions, and other physical characteristics would be affected in any way. Furthermore, in view of the fact that SPS has the ability to inactivate certain antibiotics (3, 5), additional experiments were performed to determine if SPS present in the sheep blood would give aberrant zone sizes on antibiotic susceptibility testing plates which incorporate sheep blood for the growth of fastidious organisms.

MATERIALS AND METHODS

Blood collection with SPS. Blood was drawn from sheep which had been fasted for 18 to 24 h. The neck area was shaved and swabbed with tincture of iodine. A venipuncture was performed in the jugular vein using a 36-inch blood collection set (Abbott Laboratories). The blood was collected in a Becton Dickinson Vacutainer bottle containing 1 ml of 5% SPS (Roche) for each 100 ml of blood to be drawn. The bottle was inverted occasionally to facilitate mixing; no clotting has been found in more than 100 liters of blood drawn in this manner.

When the blood arrived in the laboratory, a 15-ml sample was removed to determine the hematocrit and to check for sterility. The remaining blood was stored at 4 C prior to use. Sterility testing was performed by inoculating 5 ml of blood into two evacuated bottles containing 50 ml of Columbia broth (Hospital Service Technology). One bottle was vented and provided an aerobic atmosphere whereas the other bottle was left anaerobic. The two bottles were incubated at 37 C for 7 days and checked daily for visible evidence of microbial growth, prior to use.

Defibrinated blood collection. Blood was collected by the venipuncture method described above. The collection vessel was a 1,000-ml Erlenmeyer flask fitted with a rubber stopper. Two layers of glass beads (5 mm diameter) were used to cover the bottom of the flask. The flask, stopper, and glass beads were autoclaved together prior to use. A vacuum was established in the flask by inserting an 18-gauge needle fitted with thick-walled rubber tubing into the stopper, and connecting the other end of the tubing to a vacuum pump. Blood was drawn with a collection set

as described above. The flask was rotated manually in a circular motion at approximately 150 to 200 rpm. The blood, approximately 300 to 400 ml, was collected until there were no longer free beads in the flask. Minimal hemolysis was found if the rotation of the flask was stopped within 8 to 10 rotations after the blood flow had been stopped. Once the blood arrived in the laboratory, it was decanted into sterile storage bottles. A 15-ml sample of this blood was used in performing a hematocrit and for sterility testing as described previously. All defibrinated blood was also stored at 4 C prior to use.

Preparation of blood agar plates for routine use. In each case, Columbia agar base (BBL) was prepared by the manufacturer's instructions, and sufficient blood was aseptically added to the cooled agar to give a final concentration of 5%. Approximately 18 ml of blood agar was dispensed into sterile 100-mm petri dishes and allowed to harden. All prepared plates were stored at 4 C.

Preparation of blood agar plates for antibiotic susceptibility studies. The blood agar plates used for antibiotic susceptibility studies were prepared as above except that Mueller-Hinton agar (BBL) was used instead of Columbia base, and 150-mm petri dishes were used to facilitate the testing.

Preparation of bacterial isolates. Most organisms used in the study were obtained from our diagnostic laboratory (Table 1). However, a few isolates were obtained from the Center for Disease Control and College of American Pathologists proficiency testing programs. Each isolate was inoculated into 2 ml of Trypticase soy broth (BBL), and the turbidity was adjusted to the barium sulfate standard used in the Kirby-Bauer method of antibiotic susceptibility testing. A loopful from this broth was streaked onto both a defibrinated sheep blood agar plate and a SPS blood agar plate. All plates were incubated aerobically at 37 C with the exception of plates inoculated with *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis* which were placed in a CO_2 incubator. Plates inoculated with *Bacteroides fragilis*, *Clostridium perfringens*, peptostreptococcus, and peptococcus were incubated in an anaerobic GasPak jar (BBL). All plates were examined by the same technologist after 24 h of incubation, except fungal isolates which were examined after 1 week. The technologist did not know the source of the blood used in the plate. Quality of growth, pigment production, hemolytic reactions, and ability to swarm were compared in each case and recorded. Tests for inhibition of growth using Taxo A and Taxo P disks (BBL), as well as bile solubility with deoxycholate reagent (BBL), were also examined with appropriate organisms by manufacturer's instructions.

Protocol for antibiotic susceptibility testing. Two organisms were chosen representing the two most common types of organisms that would normally require 5% sheep blood for antibiotic susceptibility testing. These organisms were *B. fragilis* and *Streptococcus faecalis*. Inocula were prepared by the Kirby-Bauer protocol and the plates were incubated in anaerobic and aerobic atmospheres, respectively. We realize that the Kirby-Bauer system is not acceptable for anaerobic organisms; however, its use here was specifically for comparing the effect of the media on zone sizes only. Since some difference in zone sizes is inevitable even using the same media, it was decided to test each organism against the antibiotics 10 times on both media concurrently. The same technologist measured and recorded all zone sizes. A two-factor computerized analysis of variance (6) was performed using the 10 zone sizes for each antibiotic from each type of media to determine if any differences in the zone sizes were the result of using the different blood preparations.

RESULTS

Concurrently, 117 strains of bacteria and fungi were tested on both 5% defibrinated and

TABLE 1. *Bacterial and fungal isolates tested on both defibrinated and SPS blood agar plates*

Organism	No. tested	Organism	No. tested
Acinetobacter calcoaceticus	2	*Histoplasma capsulatum*	1
A. lwoffi	3	*Listeria monocytogenes*	1
Actinobacillus lignierisi	1	*Neisseria gonorrhoeae*	2
Aeromonas hydrophila	2	*N. meningitidis*	4
Bacteroides fragilis	5	*Peptococcus* spp.	2
Blastomyces dermatitidis	1	*Peptostreptococcus* spp.	8
Bordetella bronchiseptias	1	*Proteus mirabilis*	3
Candida albicans	4	*Providence stuartii*	4
Citrobacter freundii	3	*Pseudomonas aeruginosa*	7
Clostridium perfringens	3	*Salmonella* spp.	4
Corynebacterium diphtheriae	1	*Serratia marcescens*	4
Edwardsiella tarda	1	*Shigella sonnei*	4
Enterobacter cloacae	4	*Staphylococcus aureus*	6
Escherichia coli	7	*Streptococcus faecalis*	4
HB-1	1	*S. pneumoniae*	4
Haemophilus influenzae[a]	4	*S. pyogenes*	4
Klebsiella pneumoniae	8	*Viridans* group *streptococcus*	4

[a] Satellite growth around *Staphylococcus aureus* streak.

SPS blood agar plates.

No physical differences could be detected between the plates, except for three strains of peptostreptococci, two of which grew slightly better on the defibrinated plates, and one strain grew slightly better on the SPS plate. All organisms tested grew well on both types of blood agar. Inhibition of growth of *Streptococcus pyogenes* with Taxo A disks as well as inhibition of growth of *Streptococcus pneumoniae* with Taxo P disks and bile solubility were demonstrated equally well using both kinds of blood agar.

Characteristic alpha, beta, and gamma hemolysis was seen with viridans group streptococci, *S. pyogenes* and *S. faecalis*, respectively. Other organisms demonstrated various hemolytic reactions, but in each case the result was the same for the two types of media. Of note was the familiar double zone of hemolysis seen with isolates of *C. perfringens* and the thin zone of beta hemolysis around colonies of *Listeria monocytogenes*. Pigmented strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* developed typical pigment on each type of medium. In addition, strains of *Proteus mirabilis* swarmed in the usual fashion on both media. Although two strains of *N. gonorrhoeae* did grow well on the blood agar plates, a chocolate agar plate would be more appropriate for routine culture of this organism.

Table 2 shows the results of testing 11 commonly used antibiotics against a single isolate of *S. faecalis* and 7 antibiotics against *B. fragilis* on both types of blood agar plates. Each organism was tested 10 times on each medium and the results were subjected to a two-factor computerized analysis of variance. In each instance, no significant difference ($P > 0.1$) was found in the zone sizes, for the antibiotics tested, between the two types of media.

DISCUSSION

Our prime objective in pursuing this study was to determine if SPS, in the given concentration, would be detrimental to any group of microorganisms commonly isolated on blood agar. Since this particular medium is utilized in practically every aspect of bacteriology and to some degree in mycology, a large variety of organisms needed to be tested.

In Table 1, it can be seen that aerobic, anaerobic, and fastidious bacteria, as well as some representative fungi, were examined. However, in evaluating any media as basic as a blood agar plate which has been modified in some manner, other factors besides good growth must be considered. Consequently, characteristics such as hemolysis, ability to swarm, pigment production, zones of inhibition around group A streptococci and pneumococci occurring with bacitracin and optochin disks, respectively, and spot bile solubility of pneumococci were also considered important aspects of a blood agar plate.

In practically every case using the 117 isolates, the SPS blood agar plates performed as well as the defibrinated blood agar plates. In addition, since blood is commonly incorporated into Mueller-Hinton agar for antibiotic susceptibility testing, we felt that the SPS blood agar plate should be examined in this regard. The

TABLE 2. *Range and mean zone sizes obtained from 10 consecutive tests with Streptococcus faecalis and Bacteroides fragilis on defibrinated (DEF) and sodium polyanethol sulfonate (SPS) sheep blood agar plates*

Antibiotic	*Streptococcus faecalis*				*Bacteroides fragilis*			
	DEF		SPS		DEF		SPS	
	Range (mm)	Mean (mm)	Range (mm)	Mean (mm)	Range (mm)	Mean (mm)	Range (mm)	Mean (mm)
Ampicillin	24-27	25	24-26	25	11-15	13	13-14	13
Carbenicillin	NT[a]	NT	NT	NT	19-22	20	18-23	21
Cephalothin	15-18	16	16-19	17	6	6	6	6
Chloramphenicol	18-20	19	18-20	19	29-31	30	28-32	30
Erythromycin	18-20	19	19-20	19	NT	NT	NT	NT
Gentamicin	17-18	17	17-18	18	NT	NT	NT	NT
Kanamycin	15-17	16	15-17	16	NT	NT	NT	NT
Lincomycin	6	6	6	6	13-18	15	13-16	14
Methicillin	6	6	6	6	NT	NT	NT	NT
Neomycin	13-14	14	13-15	14	NT	NT	NT	NT
Penicillin	18-20	19	18-20	19	6	6	6	6
Tetracycline	10-12	11	9-12	10	16-19	17	15-19	17

[a] NT, Not tested.

Kirby-Bauer methodology utilized herein was for media comparison purposes only, and not intended to demonstrate the acceptability of this method for anaerobic organisms. Testing a strain of *S. faecalis* and *B. fragilis* against a battery of commonly used antibiotics, we found that there was no significant difference in the zone sizes between the two types of blood preparations.

Kocka et al. (4) found that 7 of 16 isolates of peptostreptococcus were inhibited by a concentration of 0.05% SPS in broth culture. In our study we noticed that two strains of peptostreptococci did exhibit slightly larger colonies on defibrinated blood agar plates, although one other strain of peptostreptococcus exhibited slightly better growth on the SPS blood agar plates. Since the concentration of SPS in the blood agar plates is considerably less than that used by Kocka and co-workers, we cannot attach much significance to the slight differences in growth exhibited by the anaerobic streptococci used in our study.

From the results presented herein, it appears that SPS blood in the concentrations used has no adverse effects on the growth characteristics of a wide variety of microorganisms, and does not produce aberrant zone sizes with antimicrobial disks. It is a more rapid and efficient method of preparing blood for bacteriological media and represents a manufacturing improvement over defibrinating procedures.

ACKNOWLEDGMENT

We graciously acknowledge Norman Conant who generously provided the cultures of *Blastomyces* and *Histoplasma*, and to Thomas Pajak who kindly performed the statistical analyses.

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